(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 December 2001 (06.12.2001)

PCT

(10) International Publication Number
WO 01/92874 A1

(51) International Patent Classification[7]: G01N 33/15, 33/50

(21) International Application Number: PCT/EP01/05749

(22) International Filing Date: 19 May 2001 (19.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

00111444.6	27 May 2000 (27.05.2000)	EP
01100298.7	4 January 2001 (04.01.2001)	EP

(71) Applicant *(for all designated States except US)*: **ARTEMIS PHARMACEUTICALS GMBH** [DE/DE]; Neurather Ring 1, 51063 Köln (DE).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **LANGHEINRICH, Ulrike** [DE/DE]; Derendinger Str. 104, 72072 Tübingen (DE).

(74) Agents: **HELBING, Jörg** et al.; Von Kreisler Selting Werner, Postfach 10 22 41, 50462 Köln (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/92874 A1

(54) Title: METHOD FOR THE IDENTIFICATION OF AGENTS AND GENES INFLUENCING CARDIOVASCULAR FUNCTION

(57) Abstract: The present invention relates to a method for the identification of agents influencing cardiovascular function utilizing teleost alevin. Said method is also applicable for the identification of genes regulating heart function.

Method for the Identification of Agents and Genes Influencing Cardiovascular Function

The present invention relates to a method for the identification of agents influencing cardiovascular function utilizing teleost alevin. Said method is also applicable for the identification of genes regulating heart function.

Summary of the Related Art

Conventional drug-finding techniques are time consuming and costly. Recently high-throughput *in vitro* screening methods became available allowing pharmaceutical testing of a great number of compounds within a short period of time (S.A. Sundberg, Curr. Opin. Biotechnol., 11(1):47-53 (2000); J. Kuhlmann, Int. J. Clin. Pharmacol. Ther., 37:575-583 (1999); J.R. Zysk et al., Comb. Chem. High Throughput Screen, 1(4) 171-183 (1998); B.A. Kenny et al., Prog. Drug Res., 51:245-269 (1998)).

The compounds identified by such screening methods are then tested in mammalian systems, such as mice or rats, which are of course far slower than *in vitro* testing methods. Recently teleosts were found to be effective animal models for mammals and humans. In particular, WO 99/42606 discloses a method for screening an agent for angiogenesis activity or cell death activity (viz. the agent to be tested is administered to a teleost (e.g., zebrafish) and a response in the teleost is detected); and U.S. Patent No. 5,565,187 discloses a method for studying capillary circulation on living salmonid or other teleosts with yolk sac attached (viz. a fluorescent dye complex and a tracer is injected into the yolk sac and the fluorescence is tracked in the systemic circulation).

However, there is still need for a reliable screening method for cardiovascular drugs.

Summary of the Invention

Surprisingly it was found that the effect of cardiovascular medicaments on the heart beat of teleost alevin can be determined just by bathing teleost alevin in a drug-containing medium and subsequent visual inspection of the heart function, e.g., via a microscope. The present invention thus provides

(1) a method for the identification of agents influencing cardiovascular function which comprises
(a) preparing a medium in which fertilized teleost alevin is bathed,
(b) adding the agent to be tested to the medium with teleost alevin and
(c) visually monitoring heart beat rate, rhythm of the heart beat, contractility of the heart and/or blood flow;
(2) a preferred embodiment of (1) above, in which prior step (b) or simultaneous with step (b) a pharmaceutically active agent, preferably having an adverse effect (relative to the desired effect of the agents to be tested) on heart beat rate, rhythm of the heart, contractility of the heart and/or blood flow, is added to the medium with teleost alevin; and
(3) a method as defined in (1) and (2) above, which is suitable for identification of genes involved in cardiovascular action.

The present invention is described in more detail below.

Brief description of the Figures

Fig. 1 shows the effects of cardiac pharmaceuticals (at a concentration of 100 µM) on the heart beat rate of 2d old zebrafish larvae.

Fig. 2 shows the effect of increasing concentrations of the Ca^{2+} channel blockers nifedipine and nimodipine on the heart beat rate of zebrafish larvae (2d post fertilization).

Fig. 3 shows the reversibility of the nifedipine-induced decrease in heart beat rate of 4d old zebrafish larvae by simultaneous addition of the Ca^{2+} channel agonist Bay K 8644.

Fig. 4 shows the effect of the Bay K 8644 on the heart beat rate of 4d old zebrafish larvae.

Fig. 5 shows re-animation of 3 d old zebrafish larvae with nifedipine-induced heart arrest by addition of Bay K 8644.

Fig. 6 shows the reversibility of the nifedipine-induced decrease of heart contractility of 3d old zebrafish larvae.

Detailed description of the Invention

The present invention provides a method by which new agents influencing cardiovascular function can be found.

"Fertilized teleost alevin" in accordance with the present invention means fertilized teleost eggs (hereinafter also shortly referred to as "teleost larvae"). "Teleosts" in accordance with the present invention include zebrafish and medaka. The preferred teleost is zebrafish.

The preparation of a medium in which fertilized teleost alevin is bathed in accordance with steps (a) of embodiment (1) of the invention comprises the provision of a suitable medium, adding the teleost alevin (preferably zebrafish larvae), and incubating the teleost alevin, preferably for 2 to 7 days at 22 to 28°C. Suitable

media for raising teleost alevin are known in the art and include low salt, buffer solutions (e.g., solutions containing less than 10 mM salts (alkaline and earth alkaline salts) and less than 20 mM buffer substance). For zebrafish larvae the most preferred medium is the so-called "embryo medium" comprising:
4.9 mM NaCl, 170 µM KCl, 329 µM $CaCl_2$, 331 µM $MgSO_4$, pH 7.2 (M. Westerfield, The zebrafish book, University of Oregon Press, Eugene, OR, USA (1993)) which is supplemented with 10 mM HEPES.

In step (b) compounds to be tested are added to the medium prepared in step (a), preferably 2 to 5 d after fertilization of the teleost alevin. The compound to be tested is preferably added at concentration of 100 nM to 100 µM, most preferably at 1-10 µM. Thereafter, preferably immediately to 48 h after addition of the compound to be tested, heart beat (rate, rhythm and contractility) and blood flow of the teleost is visually monitored, e.g., via a microscope such as a dissecting microscope.

A main advantage of teleost (and especially of zebrafish) is that the larvae can be raised in a large number and that they are transparent facilitating the microscopic inspection of the heart beat. Due to the prominent location of the heart just beneath the skin, agents acting on the heart rapidly reach their target.
Responses to the cardiac pharmaceuticals tested are similar between mammals and fish. Thus, the teleosts are extremely well suited organisms for the study of compounds acting on heart function, e.g. by performing high-throughput screening (HTS) assays.

With methods of the present invention, teleosts can be used to screen a large number of compounds for their effects on heart beat. For example, using 24 well format and manual techniques (addition

of the drug, pipetting of the larvae, microscopy) about 300 substances per day and person can be tested for their effects on the heart (2 concentrations per agent to be tested, each well containing about 10 zebrafish larvae). One particular advantage of the present invention, in comparison to cell-free or even-cell based conventional *in vitro* HTS assays, is that the agents tested act on an intact heart integrated in the whole-body physiology. In particular, drugs influencing heart beat rate, contractility, and blood flow could be found with the developed method.

The advantages in comparison to conventional drug-finding techniques are reliability, speed, and costs. Furthermore, hits arising from usual industrial high-throughput screening assays could be prioritized using teleosts in advance to experiments with mice or rats. Those hits might be substances with an expected effect on the heart function but also hits with other targets than the heart, in order to study potential side-effects (e.g., arrhythmia, bradycardia, tachycardia, cardiac failure) of these compounds.

Several common medications prolong cardiac QT intervals and in rare instances cause ventricular arrhythmia and torsade de pointes. The complications generally occur in patients taking cytochome-p450-inhibiting medications or in patients with a genetically based higher susceptibility. The antihistamines astemizole and terfenadine, the antipsychotic agent haloperidol and the gastrointestinal promotility drug cisapride have each been shown to exhibit high affinity block of HERG (human ether-a-gogo-related gene), explaining their cardiotoxic effects (see M. Taglialatela et al., Biochemical Pharmacology 55:1741-1747 (1998) and J.S. Mitcheson et al., Cellular Physiology and Biochemistry, 9:201-216 (1999)).

It was found that three day old zebrafish larvae incubated in a medium containing low doses of arrhythmia-inducing drugs

belonging to different pharmacological classes (including, but not limited to, astemizole, terfenadine, cisapride, thioridazine, haloperidol, droperidol, pimozide) very promptly induced an arrhythmic heart beat in nearly 100 % of larvae, (studied by inspecting anaesthetisized larvae with a dissecting microscope). Most often the arrhythmia observed resembles an atrioventricular block, the atrium beating twice as often as the ventricle. In some cases, dependent on the drug and its concentration the heart beat is not periodical but rather arrhythmic without an atrio-ventricular block. All drugs studied decreased the heart beat rate.

It is concluded that zebrafish is an excellent model organism to study the potential of compounds in inducing drug-acquired long QT-syndrome thereby leading to a higher safety profile of medications. Thus, the present invention also provides a simpler, more reliable, faster and cheaper assay for HERG-binding substances. The advantage is that this method enables the early identification of proarrhythmic drugs and thus minimizes the risk that at a late stage drug development has to be stopped.

In a preferred embodiment, for the simulation of diseases, and/or to thereby increase the responsiveness of teleosts to the agents applied (or even rendering them responsive), in step (b) – prior to or simultaneous with the addition of the agent to be tested – a pharmaceutically active agent (hereinafter "sensitizing agent") is added to the medium, for example Ca^{2+} channel blockers or agents causing arrhythmia, in order to find substances reverting the effects of the sensitizing agent and thereby rescue the larvae.

In untreated wildtype larvae, heart beat rate and contractility is fast and strong, such that an increase in contractility and/or beat rate is rather difficult to detect by microscopic inspection. However, the addition of Ca^{2+} channel blockers, e.g. nifedipine, which lowers heart beat rate and contractility, prior to or simultaneous with an

agent-containing medium, may be used to detect those agents which lead to an increase in contractility of the teleost heart (putative drugs for the treatment of congestive heart failure).
Similarely, putative anti-arrhythmic agents can be identified by studying heart beats of teleost larvae which were bathed prior to or simultaneous with the addition of the agents in a solution of an arrhythmia-inducing drug.

Thereby human diseases can be simulated (e.g., congestive heart failure or arrhythmia). Thus, the drug-treated teleost present the first whole-animal disease model, which is suited for high-throughput screenings. Using high concentrations of nifedipine (e.g. 50 µM) an arrest of the heart beat can be induced in teleost larvae, thereby offering the possibility to screen for compounds which can re-animate the heart to beat, potential drugs for the treatment of cardiac infarction.

The method of the present invention, e.g., application of Ca^{2+} channel blockers to the teleost larvae, is moreover suitable for the identification of special genes involved in cardiovascular function. These genes are expected to encode especially those proteins, which will, after applying specific antagonists, lead to an increase in cardiac contractility. Up to now, no method exists, which could yield a similar number of new genes of this specific type. By performing high-throughput screening assays with these new proteins, antagonists (medicaments) for the treatment of congestive heart failure could be found.

In accordance with said embodiment of the present invention, teleost larvae, preferably zebrafish larvae carrying hetero- or homozygous mutations are sensitized by adding for example Ca^{2+} channel blocker (step (a) of the screening method). Such mutations can, e.g., be induced by ethylnitrosurea (P. Haffter et al.,

Development 1996, 123, 1-36) or insertion mutagenesis (A. Amsterdam et al., Genes Dev., 13(20):2713-24 (1999)).

By performing a dominant or recessive genetic screen in accordance with the method of the invention and by applying Ca^{2+} channel blockers to the teleost larvae carrying mutations, phenotypes which show resistance to the drug applied can be identified. This means, that the heart beat and blood flow is less influenced by the Ca^{2+} channel blocker than in wildtypes. It is believed that these phenotypes carry mutations in genes encoding proteins, which will, after applying specific antagonists, lead to an increase in cardiac contractility. The reason for this feature is, that the underlying mutations are quite likely "loss of function" mutations ("gain of function" mutations are quite rare). Thus, with this kind of screen new targets (for which antagonists could be developed) for the treatment of congestive heart failure can be found, e.g. after positional cloning of the mutations.

Genotypes with the above mentioned mutations do not show easily identifiable phenotypes if not previously incubated with, for example, Ca^{2+} channel blockers, and are likely to be overlooked in conventional genetic screens for the heart beat (zebrafish, medaka, mice screens), because contractility is near the optimum. This conclusion is supported by the observation that an increase in cytosolic calcium induced by the Ca^{2+} channel agonist (+/-)Bay K 8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2'-(trifluoromethyl) phenyl]-3-pyridinecarboxylic acid methyl ester) has nearly no effect on cardiac performance of untreated fish, in contrast to its effects on nifedipine-treated larvae. Therefore, for getting not only genotypes but also phenotypes in a genetic screen for the contractility, the induction of a "disease status", (lowering the contractility by addition of the Ca^{2+} channel blocker) in the organism prior to screening will be of great advantage.

Since the pharmaceutical industry is much more interested (due to a higher success rate) in targets for which antagonists rather than agonists have to be developed, the described invention represents a major further development of conventional screens for contractility, which would yield mainly targets for agonists (for the treatment of heart failure).

Loss of function mutations in genes, encoding proteins, which after inhibition, cause an increase in contractility, will probably also have no obvious phenotypes in humans. Thus, screening families with heart defects and also differential display techniques are not likely to reveal these interesting targets.

The invention is further explained by the following non-limitative examples.

Examples

Material and Methods

- zebrafish larvae
- embryo medium 4.9 mM NaCl, 170 µM KCl, 329 µM $CaCl_2$, 331 µM $MgSO_4$, 10 mM Hepes, pH 7.2 (Sigma, p.a. grade)
- DMSO (Sigma; ultrapure)
- MESAB solution (0.4 % ethyl-m-aminobenzoate methane-sulfonate + 1 % Na_2HPO_4 x 2 H_2O, pH 7.2)
- Ca^{2+} channel inhibitor, for example nifedipine or nimodipine (Sigma)
- petri dishes (10 cm and 4 cm in diameter) (Greiner, Nürtingen)
- 50 ml plastic tubes (screwcaps) (Greiner, Nürtingen)
- dissecting microscope (MZ-FL III; Leica)
- incubator (Heraeus)
- timer

Example 1

Zebrafish larvae were raised according to established protocols (M. Westerfield, The zebrafish book, University of Oregon Press, Eugene, OR, USA (1993)).

About 50 zebrafish larvae were incubated for 2-7 d at 22-28°C in petri dishes filled with 30 ml embryo medium.

At the day of the experiment 10 larvae were transferred to small petri dishes filled with 10 ml embryo medium, 0.5 ml MESAB solution and 10 µl of the respective drug (or + 10 µl of a second drug) added from a 1000-fold stock solution prepared with DMSO, these three components were previously mixed in 50 ml plastic tubes (controls only receive 10 µl DMSO).

At certain time points the contractility of the heart and blood flow was monitored, and the heart rate was determined by counting the heart beat with the aid of a stereo-microscope.

Several pharmaceuticals known to have an effect on heart beat rate and/or contractility in mammals were tested. The ß-adrenergic blocker propanolol and the Ca^{2+} channel blockers verapamil (Fig. 1), nifedipine and nimodipine (Fig. 2), induced a decrease in heart beat rate, contractility and blood flow, similar to rodents and humans. The drugs propafenone (sodium channel blocker) and amiodarone (potassium channel blocker) have similar effects as the drugs mentioned above, indicating similarities between mammalian and teleost cardiac channels (Fig. 1). Bay K 8644, a Ca^{2+} channel agonist, acting on dihydropyridine-sensitive Ca^{2+} channels in mammals, led to a strong increase in contractility of nifedipine-treated zebrafish, in contrast to untreated fish (Fig. 3 and 4). Thus, the zebrafish heart reacts in a similar way (also with respect to the concentrations of the drugs and reaction time) as isolated mammalian cardiomyocytes. This feature shows, that zebrafish with artificially reduced contractility can be used for screens aimed at finding agents increasing the contractility of the heart.

Example 2

Zebrafish larvae of the F_3 or F_2 generation carrying heterozygous or homozygous mutations (induced by ethylnitrosurea or insertional mutagenesis) are raised according to the procedures described in Example 1. A genetic screen was performed by incubating about 25 zebrafish larvae of the F_2 (dominant screen) or F_3 (recessive screen) generation per crossing for 2-7 d at 22-28°C in petri dishes filled with 30 ml embryo medium. At the day of the experiment 25 larvae were incubated with 30 ml embryo medium, 1.5 ml MESAB solution and 30 µl of the respective drug added from a 1000-fold stock solution prepared with DMSO. At certain time points the contractility of the heart, heart rate and blood flow were monitored with the aid of a stereo-microscope. In the case 25 % of the larvae inspected show resistance to the drugs applied the parents will be outcrossed to another zebrafish line (WIK) and subsequently the mutations will be cloned, e.g. by positional cloning.

It was found that low concentrations of Ca^{2+} channel inhibitors (nifedipine, nimodipine) applied to the bathing medium induced a strong decrease in heart contractility, blood flow and heart beat rate of zebrafish larvae. Fig. 2 shows dose-response curves for nimodipine and nifedipine at 2d postfertilization. The effect was reversible after washout of the drug. Even zebrafish with an arrest of the heart beat for 30 min (induced by adding 50 µM nifedipine for 90 min) could be re-animated by washout of the drug, such that the heart beat rate and contractility were nearly the same as in controls (Fig. 6). The effects of nifedipine were completely blockable by the simultaneous addition of the Ca^{2+} channel agonist Bay K 8644, strongly indicating that the observed effects of nifedipine are specific and indeed the result of an inhibition of Ca^{2+} channels of the dihydropyridine type (Fig. 3). Zebrafish larvae with an arrest of the heart beat (induced by adding 50 µM nifedipine for 1 h) can be re-

animated within minutes by addition of Bay K 8644 to the bathing solution (Fig. 5). Bay K 8644 applied alone to the surrounding medium, had nearly negligible effects on heart beat rate, contractility and blood flow (Fig. 4), indicating that a further increase in the cytosolic Ca^{2+} concentration has no consequence on these parameters.

Claims

1. A method for the identification of agents influencing cardiovascular function which comprises
(a) preparing a medium in which fertilized teleost alevin is bathed;
(b) adding the agent to be tested to the medium with teleost alevin; and
(c) visually monitoring heart beat rate, rhythm of the heart beat, contractility of the heart and/or blood flow of the teleost alevin.

2. The method of claim 1, wherein the fertilized teleost alevin are zebrafish larvae.

3. The method of claim 1 or 2, wherein step (a) comprises incubating fertilized teleost alevin for about 2 to 7 days at about 22 to 28°C.

4. The method of claims 1 to 3, wherein the agent to be tested is added at a concentration of about 100 nM to about 100 µM, preferably about 1 to 10 µM.

5. The method of claims 1 to 4, wherein the heart beat rate, rhythm of the heart beat, contractility of the heart and/or blood flow are monitored with a microscope, preferably a dissecting microscope.

6. The method of claim 5 which is suitable for identification of proarrhythmic drugs.

7. The method of claims 1 to 6, wherein prior to step (b) or simultaneous with step (b) a pharmaceutically active agent (sensitizing agent), preferably a sensitizing agent having an

adverse effect (relative to the desired effect of the agent to be tested) on heart beat rate, blood flow and/or contractility in mammals, is added to the medium with zebrafish larvae.

8. The method of claim 7, wherein the sensitizing agent is added at a concentration of about 1 to 10 µM.

9. The method of claim 7 or 8, wherein the sensitizing agent is selected for example from ß-adrenergic blockers, Ca^{2+} channel blockers, sodium channel blockers or potassium channel blockers.

10. The method of claims 1 to 9, which is suitable for the identification of genes involved in cardiovascular action.

11. The method of claim 10, wherein the genes encode proteins leading to an increase in cardiac contractility.

12. The method of claims 10 or 11, wherein the teleost alevin carries heterozygous or homozygous mutations.

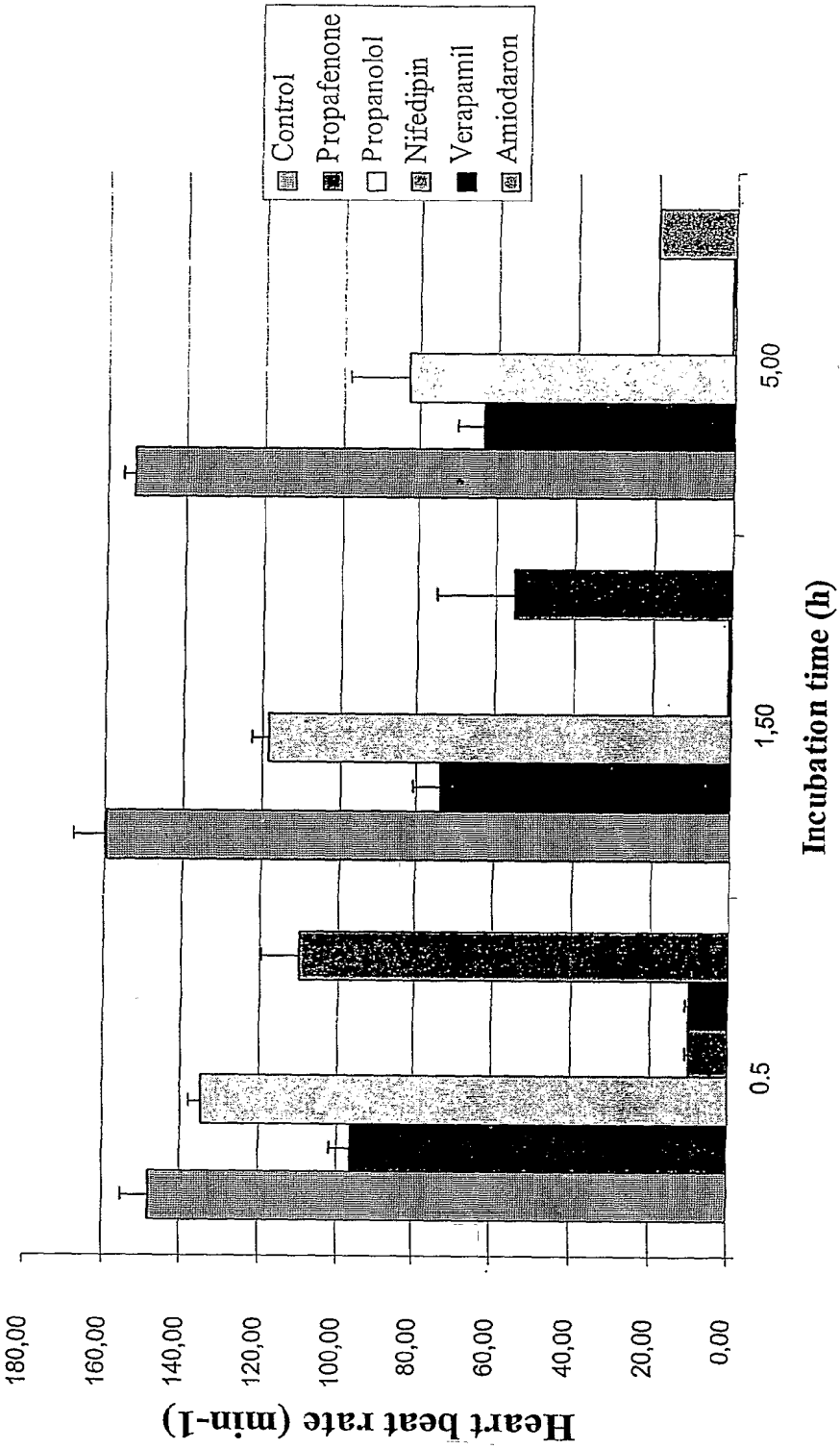
Control
Propafenone
Propanolol
Nifedipin
Verapamil
Amiodaron
Heart beat rate (min-1)
180,00
160,00
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
0.5
1,50
5,00
Incubation time (h)

Fig. 1.

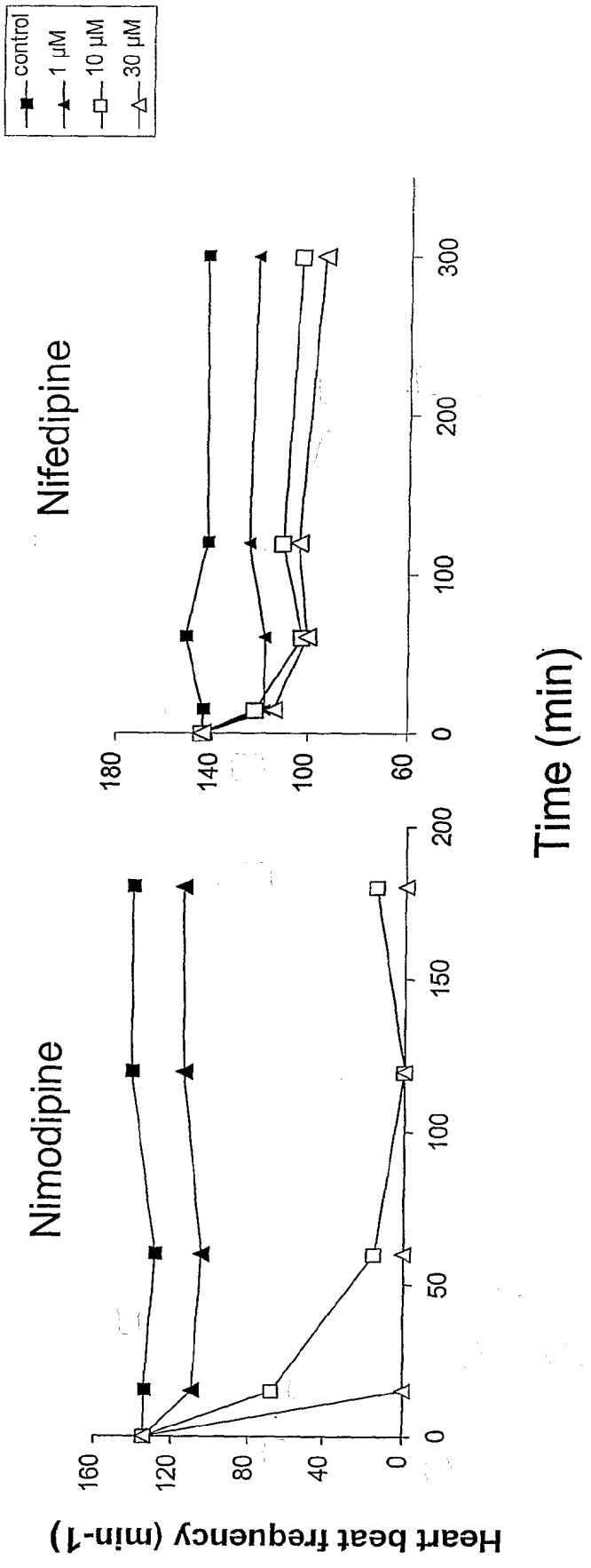
control
1 µM
10 µM
30 µM
Nifedipine
Nimodipine
Heart beat frequency (min-1)
Time (min)
160
120
80
40
0
180
140
100
60
0
50
100
150
200
300

Fig. 2

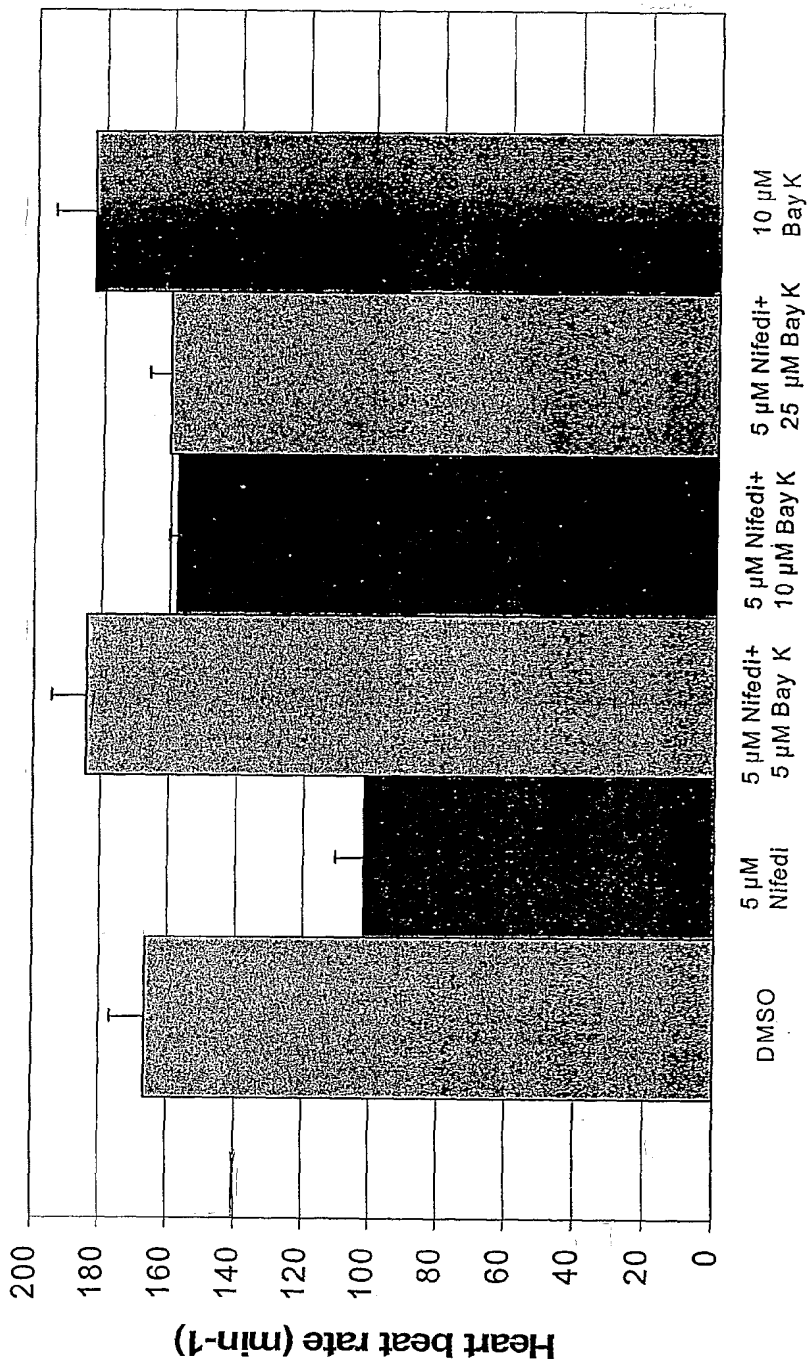
Heart beat rate (min-1)
200
180
160
140
120
100
80
60
40
20
0
DMSO
5 µM Nifedi
5 µM Nifedi+ 5 µM Bay K
5 µM Nifedi+ 10 µM Bay K
5 µM Nifedi+ 25 µM Bay K
10 µM Bay K

Fig. 3

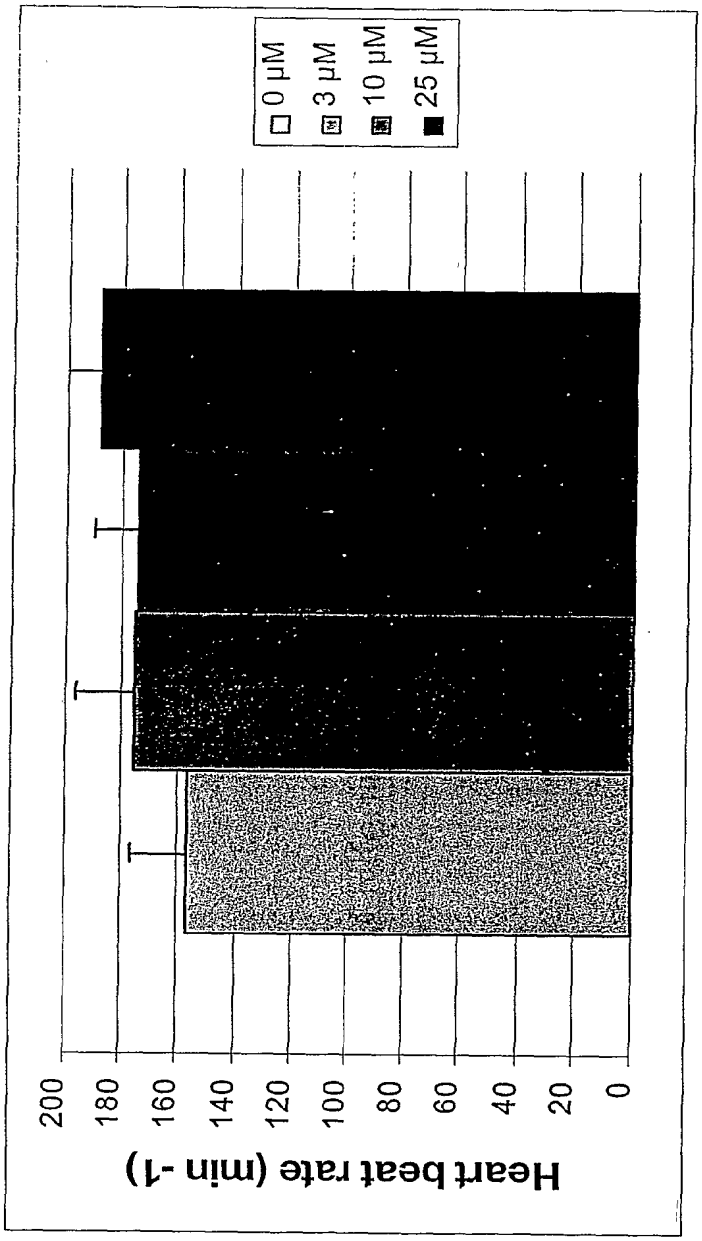
0 µM
3 µM
10 µM
25 µM
Heart beat rate (min -1)
200
180
160
140
120
100
80
60
40
20
0

Fig. 4

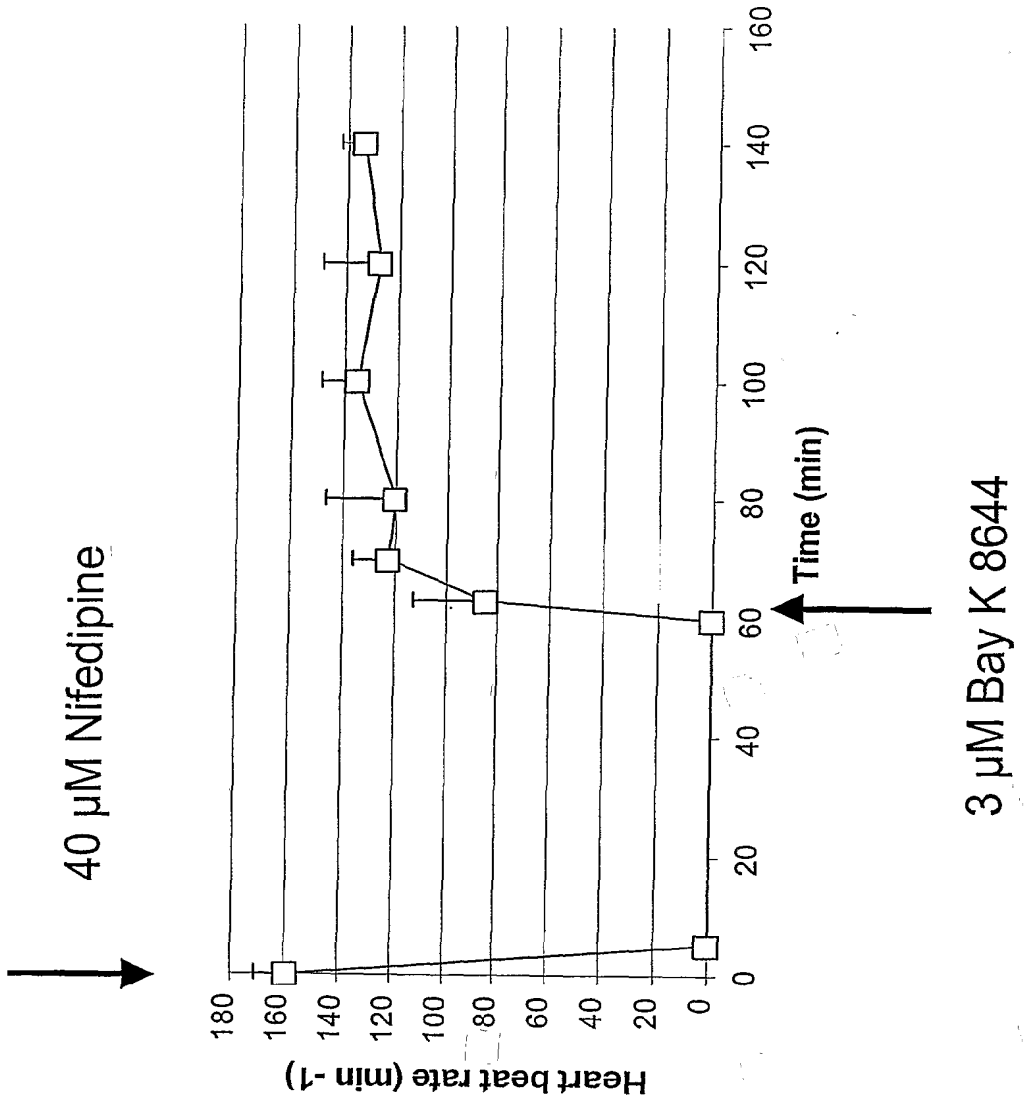
40 µM Nifedipine
3 µM Bay K 8644
Heart beat rate (min -1)
Time (min)
0
20
40
60
80
100
120
140
160
180
0
20
40
60
80
100
120
140
160

Fig. 5

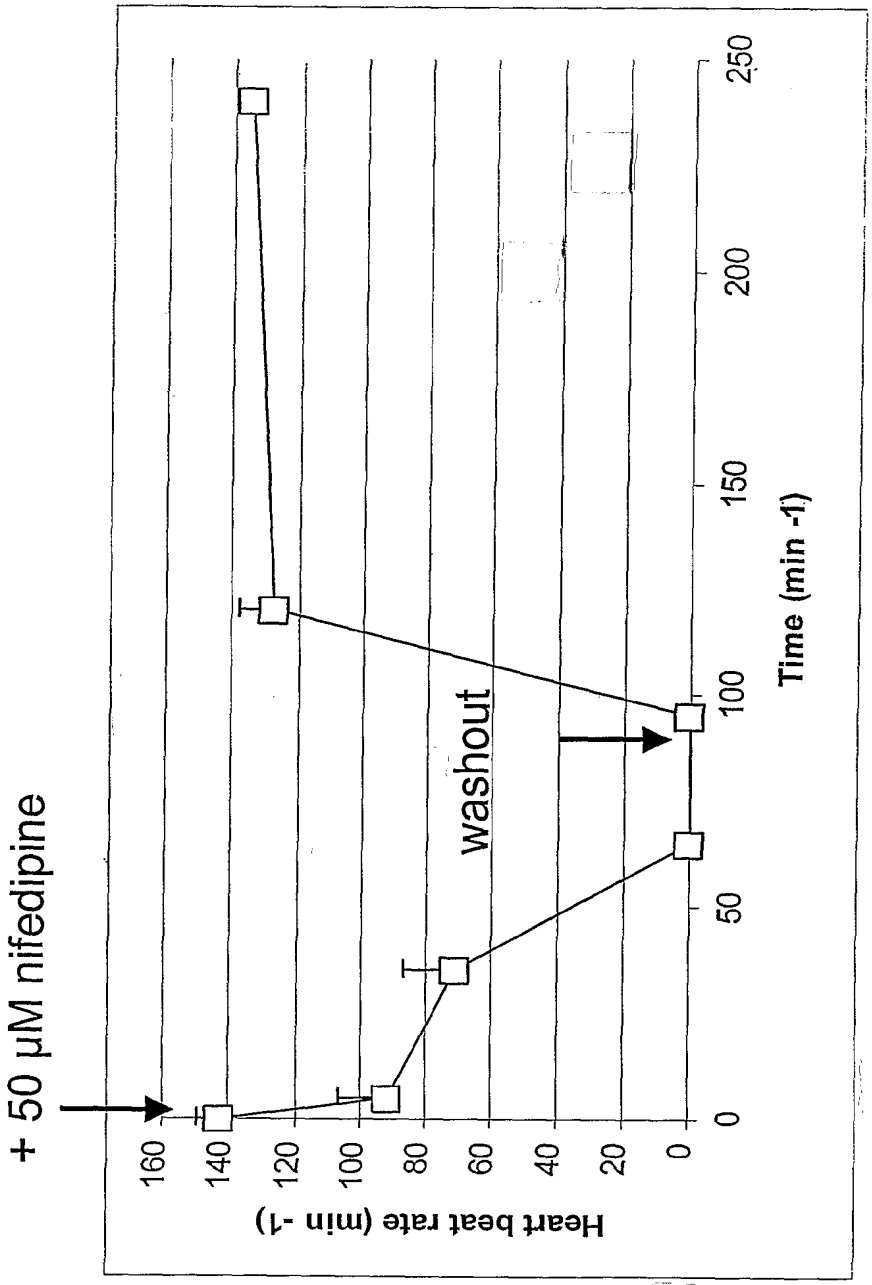
+ 50 µM nifedipine
washout
Heart beat rate (min -1)
Time (min -1)
0
20
40
60
80
100
120
140
160
0
50
100
150
200
250

Fig. 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/05749

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/15 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, MEDLINE, CHEM ABS Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 42606 A (SERBEDZIJA GEORGE N ;SEMINO CARLOS (US); FROST DEANNA (US); PHYLON) 26 August 1999 (1999-08-26) cited in the application the whole document	1-5
A	WO 98 31787 A (LEON GATCHALIAN CHRISTINE DE ;EISAI CO LTD (JP); RUBIN LEE L (US)) 23 July 1998 (1998-07-23) the whole document	1-12
A	US 5 932 418 A (YAGER THOMAS DEAN) 3 August 1999 (1999-08-03) the whole document	1-4

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2001	22/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Luzzatto, E

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/05749

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 565 187 A (ZIKRIA BASHIR ET AL) 15 October 1996 (1996-10-15) cited in the application the whole document -----	1-12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 01/05749

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9942606 A	26-08-1999	AU 3307499 A	06-09-1999
		EP 1066402 A	10-01-2001
WO 9831787 A	23-07-1998	EP 0964915 A	22-12-1999
US 5932418 A	03-08-1999	NONE	
US 5565187 A	15-10-1996	NONE	